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Short Communication

Combined action of type I and type III interferon restricts initial replication of severe acute respiratory syndrome coronavirus in the lung but fails to inhibit systemic virus spread

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STAT1-deficient mice are more susceptible to infection with severe acute respiratory syndrome coronavirus (SARS-CoV) than type I interferon (IFN) receptor-deficient mice. We used mice lacking functional receptors for both type I and type III IFN (double knockout, dKO) to evaluate the possibility that type III IFN plays a decisive role in SARS-CoV protection. We found that viral peak titres in lungs of dKO and STAT1-deficient mice were similar, but significantly higher than in wild-type mice. The kinetics of viral clearance from the lung were also comparable in dKO and STAT1-deficient mice. Surprisingly, however, infected dKO mice remained healthy, whereas infected STAT1-deficient mice developed liver pathology and eventually succumbed to neurological disease. Our data suggest that the failure of STAT1-deficient mice to control initial SARS-CoV replication efficiently in the lung is due to impaired type I and type III IFN signalling, whereas the failure to control subsequent systemic viral spread is due to unrelated defects in STAT1-deficient mice.

Severe acute respiratory syndrome coronavirus (SARS-CoV) is a highly pathogenic zoonotic virus that emerged in China in late 2002 (Drosten *et al.*, 2003; Skowronski *et al.*, 2005). It spread quickly to 29 countries worldwide, infecting more than 8000 people with a case fatality rate of approximately 10 % (WHO; http://www.who.int/csr/sars/country/table2004_04_21/en/index.html). Although the disease was characterized primarily as atypical pneumonia, extrapulmonary viral spread associated with gastrointestinal symptoms and hepatitis were also observed (Ding *et al.*, 2003; Leung *et al.*, 2003; Peiris *et al.*, 2003a, b; Farcas *et al.*, 2005).

Human isolates of SARS-CoV do not cause overt illness or pathology in commonly used mouse strains (Glass *et al.*, 2004; Hogan *et al.*, 2004). However, a mouse-adapted SARS-CoV variant, designated rMA15, closely mimics the virus-induced illness seen in SARS patients when used for infection of BALB/c mice (Roberts *et al.*, 2007). rMA15 leads to rapid death in BALB/c mice due to high virus replication and severe lung damage. Infection of BALB/c mice with rMA15 further results in uncontrolled extrapulmonary virus spread. In contrast, mice of 129 or C57BL/6 genetic background support initial pulmonary replication of rMA15 but clear the infection within 1 week, indicating that undefined host factors greatly influence resistance towards SARS-CoV (Frieman *et al.*, 2010; Sheahan *et al.*, 2008).

Interferons (IFNs) are cytokines that play a crucial role in immune responses against many viruses (Haller *et al.*, 2006). Upon viral infection, rapid production of type I and type III IFN not only initiates the antiviral state, but also modulates adaptive immunity (Le Bon & Tough, 2002; Morrow *et al.*, 2009). Type I IFN acts through a ubiquitous heterodimeric receptor complex consisting of alpha/beta interferon receptor (IFNAR)1 and IFNAR2 subunits (Uzé *et al.*, 2007). Binding of type I IFN to its receptor activates Jak1 and Tyk2 kinases that phosphorylate STAT1 and STAT2 transcription factors, which in turn induce the expression of more than 300 IFN-stimulated genes, many of which mediate antiviral immunity (Sadler & Williams, 2008; Ank *et al.*, 2006). Type III IFN (also designated lambda interferon, IFN-λ) mediates protection of epithelial surfaces against respiratory and gastrointestinal viruses (Mordstein *et al.*, 2010; Pott *et al.*, 2011). Despite using a distinct receptor complex consisting of interleukin 28 receptor (IL28R)1 and interleukin 10 receptor (IL10R)2

chains, type III IFN also triggers the activation of the Jak/STAT pathway like type I IFN (Kotenko *et al.*, 2003; Zhou *et al.*, 2007).

A protective role of type I IFN during SARS-CoV infection has been described *in vitro* (Cinatl *et al.*, 2003; Zheng *et al.*, 2004) and *in vivo* (Haagmans *et al.*, 2004). Retrospectively, it is also assumed that type I IFN influenced the virus-induced pathology in SARS patients (Haagmans *et al.*, 2004; Hensley *et al.*, 2004). STAT1-deficient 129 mice, which lack responsiveness to all IFN subtypes, are more susceptible to SARS-CoV-induced disease than wild-type (wt) mice (Frieman *et al.*, 2010; Hogan *et al.*, 2004). In STAT1-deficient animals, the virus was not contained in the lung as in wt mice but rather spread systemically, strongly suggesting a protective role of IFN. However, assessing the roles of the various IFN types more precisely proved difficult. Using single- and double-knockout (dKO) mice for type I or type III IFN receptors, we recently demonstrated that both of these IFN types reduce SARS-CoV titres in the lung and, acting in concert, prevent viral spread to the gastrointestinal tract (Mordstein *et al.*, 2010). Others found that the mouse-adapted SARS-CoV variant rMA15 did not induce disease in IFNAR1-deficient 129 mice that were treated with high doses of an antibody (anti-IL28R1) that can block the type III IFN receptor (Frieman *et al.*, 2010). Further, unlike STAT1-deficient mice, anti-IL28R1-treated IFNAR1-deficient mice cleared the virus efficiently from the lung, suggesting that SARS-CoV pathogenesis is regulated by a STAT1-dependent but type I and III IFN-independent mechanism (Frieman *et al.*, 2010). However, these experiments could not exclude the possibility that the antibody treatment had failed to completely neutralize the virus-induced type III IFN.

To evaluate this possibility, we performed a side-by-side comparison of STAT1$^{-/-}$ mice and IFNAR$^{-/-}$IL28R$^{-/-}$ dKO mice that lack functional receptors for both type I and type III IFN (Mordstein *et al.*, 2008). Both of these mutant mouse strains were of identical C57BL/6 genetic background, thus excluding the possibility that differences in virus susceptibility are simply due to other genetic factors. Infection with rMA15 virus was done via the intranasal route, and virus titres in the lung were measured at the indicated times post-infection (p.i.) by plaque assay on Vero cells. Lung virus titres peaked on day 2 p.i. in all mouse strains, but wt animals contained significantly less infectious virus in the lung than dKO or STAT1$^{-/-}$ animals (Fig. 1a). Virus titres decreased gradually to undetectable levels by day 7 p.i. in wt animals and by day 9 p.i. in dKO and STAT1$^{-/-}$ animals. Importantly, dKO and STAT1$^{-/-}$ animals displayed comparable lung virus titres on all days examined, although these were significantly higher than lung virus titres in wt animals. These data were supported by viral RNA quantification using real-time RT-PCR (qRT-PCR), which showed that viral RNA loads in the lungs on days 2 and 5 p.i. were similar in dKO and STAT1$^{-/-}$ animals, but significantly higher than in wt animals (Fig. 1b). These results demonstrated that the simultaneous absence of type I and III IFN mimicked the situation in STAT1$^{-/-}$ animals, indicating that these two IFN subtypes play a role in defence against SARS-CoV.

Although dKO and STAT1$^{-/-}$ animals both cleared infectious virus from the lung by day 9 p.i., STAT1-deficient

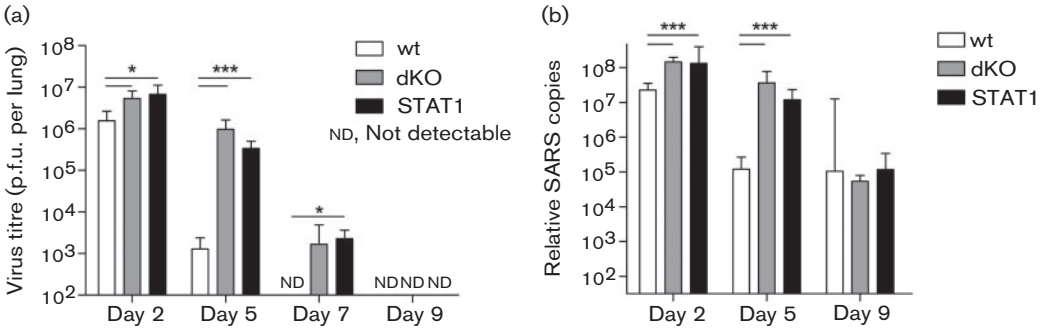

Fig. 1. Combined action of type I and type III IFNs restricts SARS-CoV infection in the lung. (a) Groups (n=5–6) of 8-week-old C57BL/6 (wt), IFNAR$^{-/-}$IL28R$^{-/-}$ (dKO) and STAT1$^{-/-}$ mice were infected intranasally with 1×10^5 p.f.u. of mouse-adapted SARS-CoV strain rMA15. At indicated time points p.i., mice were sacrificed and the lungs were homogenized in 800 µl cold PBS containing 0.3 % BSA using a FastPrep 24 instrument (MP Biomedicals). Homogenates were spun at 2500 ***g*** for 5 min and the cleared supernatants were used for virus titration by conventional plaque assay on Vero cells. (b) The number of viral genome copies was determined by qRT-PCR. Total RNA was extracted from lung homogenates of the above-described animals with peqGOLD TriFast reagent (Peqlab) and first-strand cDNA synthesized using a Revertaid first-strand cDNA synthesis kit (Fermentas). Taqman real-time PCR was performed using primers and probes detecting ORF1B sequences of SARS-CoV as described earlier (Drosten *et al.*, 2003). Values presented are relative viral RNA levels normalized to endogenous β-actin control with the x-axis set to the detection limit (Radonić *et al.*, 2004). Values shown are means ± SD. Asterisks indicate statistically significant differences: *$P<0.05$, ***$P<0.001$.

but not dKO mice showed mild signs of disease, including hunched posture and ruffled fur, during the second week of infection. Substantial liver pathology was observed in STAT1$^{-/-}$ animals in the form of white macroscopic nodules (Fig. 2a) that were not observed in dKO animals. Haematoxylin and eosin (H&E) staining of liver sections identified these nodules as massive infiltration of mononuclear inflammatory cells (Fig. 2b), as reported previously by others (Frieman *et al.*, 2010; Hogan *et al.*, 2004). Virus isolation from the liver and the small intestine of STAT1$^{-/-}$ mice was unsuccessful, although high levels of viral RNA were detected in these organs on day 9 p.i. (Fig. 2c). In contrast, only traces of viral RNA were detected in the intestine and liver of dKO mice. Positive immunostaining with a SARS-CoV nucleocapsid (N) protein-specific polyclonal rabbit antiserum in inflammatory nodules of the liver suggested that the observed pathology in STAT1$^{-/-}$ mice was induced by SARS-CoV (Fig. 2d).

To follow the progression of liver disease in STAT1$^{-/-}$ mice, we infected groups of STAT1$^{-/-}$ and dKO mice, and observed the animals over a period of 42 days. Starting at about day 20 p.i., infected STAT1$^{-/-}$ animals began to develop paralysis of the hindlimbs. These symptoms frequently coincided with severe weight loss. Upon loss of >25 % of initial body weight or severe paralysis of a limb, the animal was euthanized and scored dead. By day 42 p.i. around 70 % of the infected STAT1$^{-/-}$ mice had succumbed to disease (Fig. 3a). Organ samples were collected from all deceased animals and from animals that survived until the end of the experiment on day 42 p.i. By qRT-PCR, 12 out of 18 brain samples from infected STAT1$^{-/-}$ animals were virus-positive (Fig. 3b), whereas replication-competent virus was detected in only four out of 18 brains, with a mean titre of 2.5×10^3 p.f.u. per brain (data not shown). There was no correlation between onset of disease and successful virus isolation from the brains: virus isolation was successful from the brain of two symptomatic animals sacrificed on days 20 and 38 p.i., and from the brain of two non-symptomatic animals sacrificed on days 41 and 42 p.i. In one of these brains, viral antigen could also be detected by immunostaining using a SARS-CoV N-protein-specific polyclonal rabbit antiserum (data not shown). In contrast, no viral RNA was detected in brains of infected dKO mice (Fig. 3b). Analysis of liver samples revealed the presence of viral RNA in 12 out of 17 infected STAT1$^{-/-}$ mice (Fig. 3b). In contrast, livers of infected dKO mice contained no viral RNA, suggesting that the systemic spread of SARS-CoV in STAT1-deficient mice cannot be explained solely by the lack of type I and III IFN signalling in such animals.

We demonstrated here that dKO mice, which lack functional type I and type III IFN receptors, mimic STAT1-deficient mice during the initial phase of SARS-CoV infection. Unlike wt C57BL/6 mice, dKO and STAT1-deficient animals failed to control virus replication efficiently in the lung. Peak viral titres were enhanced and the kinetics of virus clearance were delayed in both mutant mouse strains. From these observations, we concluded that SARS-CoV is sensitive to the combined action of type I and type III IFN. This conclusion is in conflict with earlier findings (Frieman *et al.*, 2010) which indicated that neither type I nor type III IFN is important for efficient control of SARS-CoV in mice. This discrepancy may be explained by the fact that Frieman and co-workers did not use knockout mice for their studies, but rather tried to block the type III IFN receptor by treating the mice with a

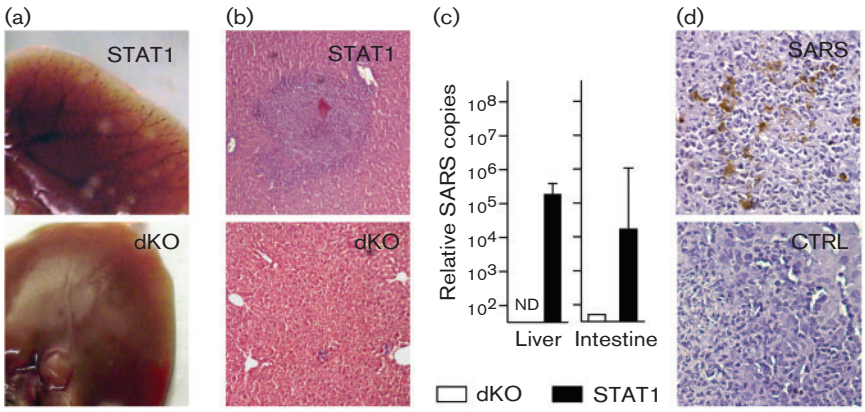

Fig. 2. Extrapulmonary spread of SARS-CoV in STAT1-deficient animals leads to liver pathology. (a) Livers from infected dKO and STAT1$^{-/-}$ animals were harvested on day 9 p.i. and photographed. Note the macroscopic nodules in the liver of STAT1$^{-/-}$ mice. (b) Inflammatory cell infiltrates observed in H&E-stained section of livers from infected STAT1$^{-/-}$ but not dKO animals at day 9 p.i. (c) Viral genome copies in STAT1$^{-/-}$ and dKO mice (n=5–6 animals per group) at day 9 p.i. determined by qRT-PCR. Values presented are means ± SD of relative viral RNA levels normalized to endogenous β-actin control as in Fig. 1(b). ND, Not detectable. (d) Liver sections of a STAT1$^{-/-}$ mouse stained with SARS-CoV N-protein-specific polyclonal rabbit serum (SARS) or control serum (CTRL). Viral antigen is stained brown in the outer regions of the inflammatory cell infiltrates.

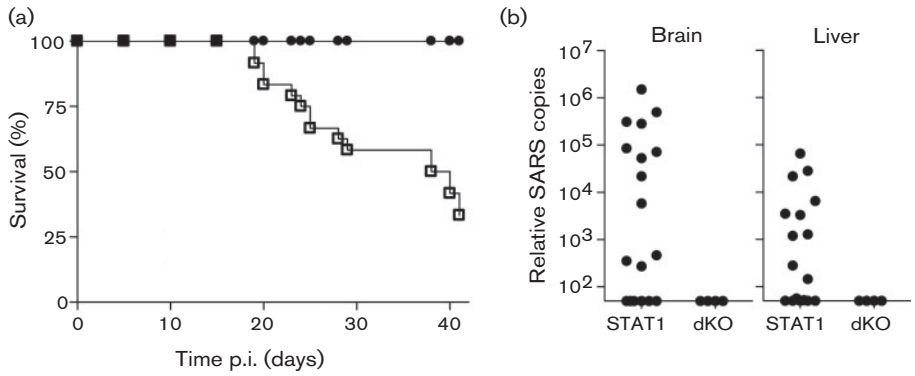

Fig. 3. STAT1-deficient animals die of a neurological disease during SARS-CoV infection. (a) dKO (●, $n=14$) and STAT1$^{-/-}$ (□, $n=24$) mice infected with SARS-CoV rMA15 were monitored daily for clinical signs of disease. The majority of STAT1$^{-/-}$ mice started to develop hindlimb paralysis by 3–7 weeks p.i. Affected animals were euthanized and scored dead. (b) Viral activity as determined by qRT-PCR in brain and liver of infected STAT1$^{-/-}$ (deceased and surviving) and dKO mice surviving until day 42, when the experiment shown in (a) was terminated. Values presented are relative viral RNA levels normalized to endogenous β-actin control as in Fig. 1(b).

receptor-specific antibody. It is conceivable that receptor blockage in lung epithelial cells is difficult to achieve by systemic antibody treatment.

Interestingly, the SARS-CoV infection had different clinical outcomes in dKO and STAT1-deficient mice. We found that rMA15 viral infection was self-limiting in dKO mice, whereas it turned into a persisting systemic infection in STAT1-deficient mice that eventually resulted in severe clinical symptoms when the virus reached the brain. These observations suggest that systemic viral spread in STAT1-deficent mice is not simply due to the impaired action of type I and type III IFN. Rather, a different STAT1-dependent control mechanism must be operative under these conditions, as concluded previously (Frieman *et al.*, 2010). Since STAT1 is also critically involved in the gamma interferon (type II IFN) signalling pathway (Aaronson & Horvath, 2002), it is tempting to speculate that the simultaneous deficiency of the type I, type II and type III IFN signalling pathways may largely determine the SARS-CoV susceptibility phenotype of STAT1-deficient mice. However, single-knockout mice with a deficient type II IFN system did not exhibit enhanced susceptibility to SARS-CoV (Frieman *et al.*, 2010). Thus, in order to test this hypothesis rigorously, triple-knockout mice would be required.

STAT1 also serves as the signal transducer of chemokine receptors involved in the recruitment of inflammatory leukocytes to the SARS-CoV-infected lung (Mellado *et al.*, 2001). In fact, mice deficient in chemokine receptors CCR1, CCR2 or CCR5 exhibit more serious disease after SARS-CoV infection than wt animals (Sheahan *et al.*, 2008). As demonstrated for another coronavirus, mouse hepatitis virus, depletion of CCR1 leads to increased mortality (Hickey *et al.*, 2007). Likewise, CCR5 is able to control West Nile virus replication in the central nervous system (CNS) and can protect from mortality (Glass *et al.*, 2005). Hence, it remains possible that the serious illness in STAT1-deficient animals was due to reduced inflammatory cell recruitment, and that the neurological symptoms in STAT1$^{-/-}$ mice are due to lack of chemokine receptor-dependent SARS-CoV control in the CNS.

Taken together, we have shown that type I and type III IFN signalling is crucial for controlling SARS-CoV replication in the lung. In contrast, extrapulmonary virus spread and virus-induced pathology at extrapulmonary sites are independent of these IFN types and rather depend on unidentified factors which require STAT1 for transcriptional activation.

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